Forest Pathology WILEY-BLACKWELL

For. Path. 43 (2013) 513–517 doi: 10.1111/efp.12058

SHORT COMMUNICATION

Development of a quantitative real-time PCR assay for the detection of *Phytophthora austrocedrae*, an emerging pathogen in Britain

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Summary

A TaqMan real-time PCR assay was developed for *Phytophthora austrocedrae,* an emerging pathogen causing severe damage to juniper in Britain. The primers amplified DNA of the target pathogen down to 1 pg of extracted DNA, in both the presence and absence of host DNA, but did not amplify any of the non-target *Phytophthora* and fungal species tested. The assay provides a useful tool for screening juniper populations for the disease.

1 Introduction

Phytophthora austrocedrae was first described in 2007 associated with mortality of Chilean cedar (*Austrocedrus chilensis)* in Argentina (Greslebin et al. 2007; Greslebin and Hansen 2010). The pathogen was not reported elsewhere until early 2011 when it was found killing Nootka cypress (*Chamaecyparis nootkatensis*) and Lawson cypress (*C. lawsoniana*) in Scotland (S. Green and G. A. MacAskill, unpublished). More recently, the pathogen has been found causing extensive dieback and mortality of juniper (*Juniperus communis*) in northern Britain (Green et al. 2012), with twelve juniper sites now confirmed as infected. In addition to these field outbreaks, DNA of *P. austrocedrae* has been identified in diseased juniper located in nurseries or private gardens in Britain (A. Schlenzig, unpublished; Denton et al. 2010).

As *P. austrocedrae* is very slow growing and difficult to isolate, diagnosis has relied on DNA extraction from diseased bark followed by sequencing of the ITS region. However, this process does not always yield a result, suggesting that conventional PCR might not be sufficiently sensitive to detect very small quantities of pathogen DNA. Given current interest in screening juniper in Britain for *P. austrocedrae*, a fast, accurate and specific diagnostic tool is needed. The aim of this study was to develop a highly sensitive TaqMan real-time PCR assay for the specific detection of *P. austrocedrae* in host tissue.

2 Materials and methods

Isolates of *P. austrocedrae* were collected from juniper at two locations in northern Britain (Table 1). Inner bark (phloem) samples from lesion margins were plated on to SMA + MRP *Phytophthora* selective medium (Brasier et al. 2005) and incubated at room temperature (15–24°C) in the dark. For sequencing, isolates were grown for 14 days in 1 ml V8 broth in an Eppendorf tube, centrifuged for 5 min at 14 000 ***g***, the supernatant was poured off, and DNA was extracted from mycelia using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). Standard PCR was performed using the *Phytophthora*-specific forward primer Ph2 (Ippolito et al. 2002) and universal reverse primer ITS4 (White et al. 1990) and the purified PCR product sequenced in both directions with the BIGDYE version 3.1 Ready Reaction Kit on an ABI PRISM 3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA). Raw sequences were aligned and edited using SEQUENCHER version 4.8 for Windows and aligned with published ITS sequences in GenBank using BLAST (Altschul et al. 1990). TaqMan primers and probe were designed using PRIMER EXPRESS version 2 (Applied Biosystems).

Real-time PCR amplification of *P. austrocedrae* DNA was performed in TaqMan Environmental Master mix 2.0 (Applied Biosystems) in 20 μl reaction volumes with primer and probe concentrations of 250 nM and a total of 2 μl template. The PCRs also included a VIC-labelled internal positive TaqMan control (Applied Biosystems), used according to the manufacturer's recommended concentrations, to detect inhibition of reactions by template DNA. The PCR was carried out in an ABI PRISM 7900HT Real-Time PCR System (Applied Biosystems) using cycle parameters of 95°C for 10 min followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 min.

Primer specificity was tested on DNA extracted from *P. austrocedrae* isolates TDJ3, TDJ6, GA3 and GAT6 (Table 1) as well as 22 other non-target species (Table 1). DNA extracted from healthy juniper and Lawson cypress was included in each series of reactions as a negative control. Primer sensitivity was tested on a serial dilution of 10 000, 1000, 100, 10 and 1 pg DNA extracted from *P. austrocedrae* isolate TDJ3. An additional dilution series was spiked with 5 ng of DNA extracted from healthy juniper. Each reaction was replicated three times within a single PCR run. For all tests, the PCR amplification efficiency (E) was calculated based on the slope of the standard curve using the equation $E = (10^{[-1/\text{slope}]} - 1) \times 100$.

The quantitative assay was also tested on DNA extracted from 65–99 mg of infected bark collected from diseased juniper, Nootka cypress and Lawson cypress trees (Table 1). Each sample was tested twice in separate PCRs with 2 μl DNA diluted 1/15 and a standard curve generated for *P. austrocedrae* isolate TJD3 containing 10 000, 1000, 100, 10 and 1 pg DNA.

Received: 1.11.2012; accepted: 3.5.2013; editor: S. Woodward

Table 1. Isolates used in this study with details of their host species, geographical location, year of collection and organization supplying the isolate.

Isolates		Host	Origin and year of collection	Source[1]	Real-time PCR specificity (+/−)
TDJ3	*Phytophthora austrocedrae*	*Juniperus communis*	Upper Teesdale, England 2011	FR, NRS	+
TDJ6	*P. austrocedrae*	*J. communis*	Upper Teesdale, England 2012	FR, NRS	+
GA3	*P. austrocedrae*	*J. communis*	Glen Artney, Scotland 2012	FR, NRS	+
GAT6	*P. austrocedrae*	*J. communis*	Glen Artney, Scotland 2012	FR, NRS	+
GA2	*Amylostereum laevigatum*	*J. communis*	Glen Artney, Scotland 2012	FR, NRS	−
2035	*Phomopsis juniperovora*	*J. communis*	Tain, Scotland 2002	FR, NRS	−
51.1	*Heterobasidion annosum*	*Picea sitchensis*	Monaughty, Scotland, year unknown	FR, NRS	−
2050	*Chondrostereum purpureum*	*Alnus* sp.	England, 1988	FR, Alice Holt	−
Fc-100	*Fusarium circinatum*	*Pinus radiata*	Spain, year unknown	UPV	−
0582/8	*Phytophthora cactorum*	*Rhododendron ponticum*	Carradale, Scotland 2009	SASA	−
1591	*Phytophthora cambivora*	*Fagus sylvatica*	Scotland 2007	SASA	−
	Phytophthora cinnamomi	Unknown	Unknown	JHI	−
887	*Phytophthora citrophthora*	*Pieris* sp.	Scotland 2004	SASA	−
	Phytophthora cryptogea	Unknown	Unknown	JHI	−
	Phytophthora drechsleri	Unknown	Unknown	JHI	−
	Phytophthora eythroseptica	Unknown	Unknown	JHI	−
	Phytophthora gonapodyides	Unknown	Unknown	JHI	−
	Phytophthora heveae	Unknown	Unknown	JHI	−
1904/22	*Phytophthora hibernalis*	*Kalmia* sp.	Ardanaiseig, Scotland 2008	SASA	−
IDA1	*Phytophthora idaei*	*Rubus idaeus*	Scotland, year unknown	JHI	−
	Phytophthora infestans	Unknown	Scotland, year unknown	JHI	−
1642/52	*Phytophthora kernoviae*	*R. ponticum*	Brodick, Scotland 2008	SASA	−
FCT1	*Phytophthora lateralis*	*Chamaecyparis lawsoniana*	Balloch, Scotland 2010	SASA	−
1945	*Phytophthora nicotianae*	Unknown	Unknown	JHI	−
BBA 2/94-IIB	*Phytophthora obscura*	Isolated from soil beneath diseased *Aesculus hippocastanum*	Germany 1994	JKI	−
	Phytophthora parasitica	*Lycopersicon esculentum*	Lanark, Scotland 2009	SASA	−
511	*Phytophthora plurivora*	*Rhododendron* 'Cosmopolitan'	Scotland 2007	SASA	−
1644	*Phytophthora pseudosyringae*	*Magnolia salicifolia*	Glenarn, Scotland 2008	SASA	−
2093/2	*Phytophthora ramorum*	*Rhododendron* sp.	Stranraer, Scotland 2009	SASA	−
SCRP333	*Phytophthora rubi*	*R. idaeus*	Scotland, year unknown	JHI	−
01037/B8	*Phytophthora syringae*	*R. ponticum*	Bathgate, Scotland 2010	SASA	−
	Pythium sylvaticum	*Pseudotsuga menziesii*	Kelso, Scotland 2003	SASA	−

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3 Results and discussion

All four *P. austrocedrae* isolates from juniper yielded a 685-bp ITS amplification product that showed 100% sequence similarity within this region and shared 99% sequence similarity with the ITS sequences of isolates of *P. austrocedrae* from Argentina. TaqMan real-time PCR primers and probe specific to *P. austrocedrae* were designed to a region of the internal ITS sequences: Paus-481-F TGGTGAACCGTAGCTGTATTTAAGC, Paus-554-R GGAACAACCGCCACTCTACTTC and Paus-507-TM TGGCATTTGAACCGRCGATGTG. The TaqMan probe was FAM labelled, with a BHQ1 Black Hole quencher (Eurofins MWG).

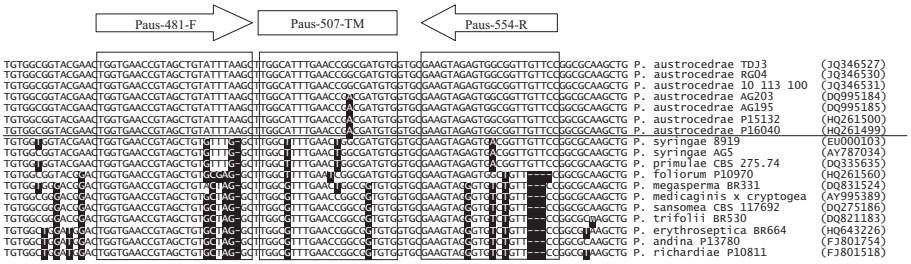

Fig. 1. ClustalW multiple sequence alignment of the ITS region of *P. austrocedrae* from Britain and Argentina and its relatives selected from the NCBI GenBank sequence database. Shaded bases denote a difference in the sequence of *P. austrocedrae* isolate TDJ3. The TaqMan real-time PCR primers and probe-binding sites used to detect and quantify infection of juniper by *P. austrocedrae* are boxed, and arrows above indicate the orientation of the primers.

Although all British *P. austrocedrae* isolates conform to a single genotype, the assay was designed with an 'R' in the probe sequence to detect both British and Argentinian genotypes of *P. austrocedrae*, which differ by a single A/G base substitution in this region (Fig. 1).

The TaqMan primers/probe amplified all isolates of *P. austrocedrae* and did not amplify DNA from any non-target species (Table 1). For the standard curves based on pure DNA extracted from *P. austrocedrae* isolate TDJ3, there was a linear relationship between cycle threshold (C_T) and the log-transformed amount of DNA (10 000–1 pg) both with and without

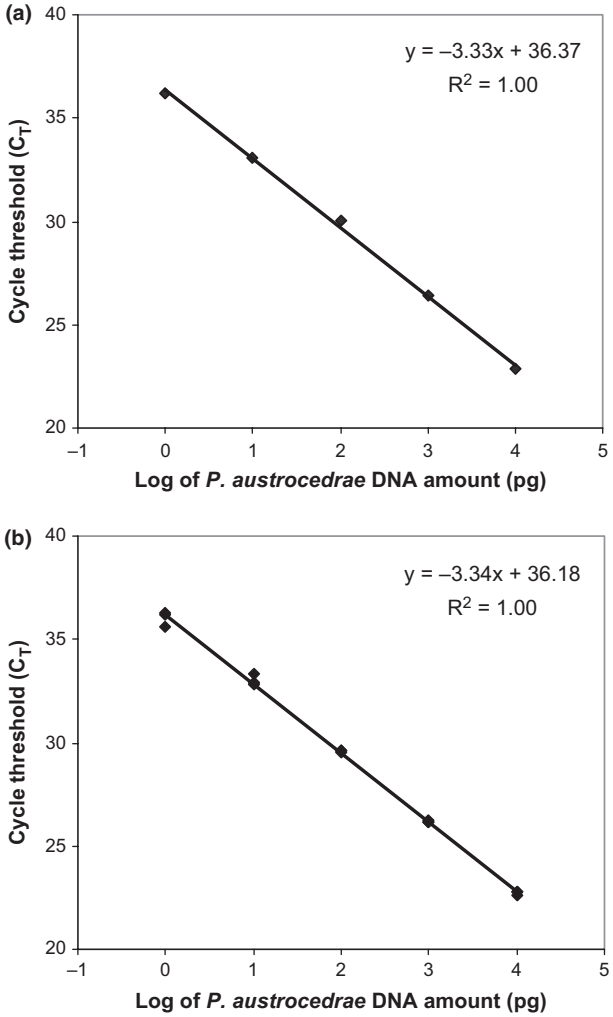

Fig. 2. Standard curves for quantifying *Phytophthora austrocedrae* DNA. (a) Graph of log-transformed DNA amounts, against cycle threshold (C_T) values, averaged over three PCR runs, with each data point in triplicate in each run. (b) Log-transformed amounts of target DNA spiked with 5 ng juniper DNA, against cycle threshold (C_T) values for three replicate reactions conducted in a single PCR run.

Table 2. Quantitative real-time PCR detection of *Phytophthora austrocedrae* in naturally infected juniper and cypress located in Britain.

Sample code	Site	Source	Conventional PCR and sequencing	*Phytophthora austrocedrae* quantification (pg)				Standard master mix IPC[1] C_T	Inhibitor-resistant master mix IPC[1] C_T
				Run 1	Run 2	Run 3	Mean		
GA1	Glen Artney 2012	Juniper bark	*P. austrocedrae*	36	34	41	37	28.62	32.20
GA2	Glen Artney 2012	Juniper bark	Not detected	0	0	0	0	28.70	31.83
GA3	Glen Artney 2012	Juniper bark	*P. austrocedrae*	502	306	233	347	29.13	31.75
GA4	Glen Artney 2012	Juniper bark	*P. austrocedrae*	518	486	481	495	29.24	31.74
GAT2	Glen Artney 2012	Juniper bark	*P. austrocedrae*	533	565	512	537	29.56	31.86
GAT3	Glen Artney 2012	Juniper bark	*P. austrocedrae*	2	2	3	2	29.41	31.87
GAT4	Glen Artney 2012	Juniper bark	*P. austrocedrae*	3	1	2	2	Undetermined	31.70
GAT5	Glen Artney 2012	Juniper bark	*P. austrocedrae*	3838	1386	1278	2168	28.64	32.00
GAT7	Glen Artney 2012	Juniper bark	*P. austrocedrae*	2230	1190	1048	1489	29.27	31.97
GAT8	Glen Artney 2012	Juniper bark	*P. austrocedrae*	1206	1216	1079	1167	29.33	31.52
GAT9	Glen Artney 2012	Juniper bark	*P. austrocedrae*	0.25	0	0.19	0.15	29.19	31.89
RG04	Glasgow 2011	Nootka cypress bark	*P. austrocedrae*	2265	1859	1856	1993	28.75	32.01
10/1113/100	Glasgow 2011	Lawson cypress bark	*P. austrocedrae*	11 242	11 355	10 410	11 002	29.75	31.86
Lesion 8a	Teesdale 2011	Juniper bark	*P. austrocedrae*	636	296	350	427	29.45	31.74
Lesion 8b	Teesdale 2011	Juniper bark	*P. austrocedrae*	249	41	41	110	29.16	31.83
TDJ8	Teesdale 2012	Juniper bark	*P. austrocedrae*	12	11	13	12	29.06	31.43
TDJ9	Teesdale 2012	Juniper bark	*P. austrocedrae*	11	11	10	10	29.21	31.73
TDJ12	Teesdale 2012	Juniper bark	*P. austrocedrae*	2	1	3	2	29.32	31.77
TDJ13	Teesdale 2012	Juniper bark	*P. austrocedrae*	6	6	7	6	29.07	31.60
HF2	Teesdale 2012	Juniper bark	*P. austrocedrae*	366	252	303	307	29.13	31.73

Quantitative data were generated from three separate runs and are shown as the total amount of target DNA in the extracts tested. An assay for inhibitory compounds in the extractions is also shown, comparing standard master mix with inhibitor-resistant master mix.
[1]Internal positive control.

the presence of 5 ng juniper DNA (Fig. 2a,b). In diseased juniper bark, *P. austrocedrae* was detected down to 1 pg DNA (Table 2). Thus, the sensitivity of the real-time PCR assay is well within the range for practical use in field-collected samples.

During preliminary testing, anomalous real-time PCR results were obtained for some bark samples when undiluted extracts were used (data not shown). For these samples, the real-time PCR worked when DNA extracts were diluted 1 in 15. To screen for inhibitors, two real-time PCR assays containing an internal positive control with either standard real-time PCR master mix (Eurogentec, Southampton, UK) or inhibitor-resistant PCR master mix (EMM 2.0; Applied Biosystems) were performed for each bark DNA extract. The results (two furthest right-hand columns in Table 2) indicate inhibition of standard master mix for sample GAT4. It is therefore recommended that diseased bark DNA samples are diluted, and an inhibitor-resistant master mix is used when performing the assay.

Acknowledgements

The authors wish to thank Dr Bridget Laue, Heather Steele and Rowan Gray for technical assistance, Dr Joan Cottrell for critical evaluation of the manuscript and Dr David Cooke for supplying isolates. This work was funded by the Forestry Commission and Scottish Government and performed under Scottish Government Plant Health Order 2005 Licence PH/4/2011.

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